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## Immunosuppressive mechanisms in glioblastoma

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Despite maximal surgical and medical therapy, the treatment of glioblastoma remains a seriously vexing problem, with median survival well under 2 years and few long-term survivors. Targeted therapy has yet to produce significant advances in treatment of these lesions in spite of advanced molecular characterization of glioblastoma and glioblastoma cancer stem cells. Recently, immunotherapy has emerged as a promising mode for some of the hardest to treat tumors, including metastatic melanoma. Although immunotherapy has been evaluated in glioblastoma in the past with limited success, better understanding of the failures of these therapies could lead to more successful treatments in the future. Furthermore, there is a persistent challenge for the use of immune therapy to treat glioblastoma secondary to the existence of redundant mechanisms of tumor-mediated immune suppression. Here we will address these mechanisms of immunosuppression in glioblastoma and therapeutic approaches.

**Keywords:** glioblastoma, immunosuppression, immunotherapy.

### Regulation of the Immune System

A variety of immune suppressive mechanisms are utilized in the setting of glioblastoma to prevent their immune detection and eradication (Fig. 1). For example, regulatory T cells (Tregs) can either originate in the thymus, and are referred to as naturally occurring Tregs (nTregs), or can be induced (iTregs) upon exposure to antigens in a tolerogenic environment. Both types of Tregs could potentially contribute to glioblastoma-mediated immune suppression, but nTregs may predominate within the glioblastoma microenvironment.<sup>1</sup> Tregs suppress immune responses by secreting cytokines, such as transforming growth factor beta (TGF- $\beta$ ) and interleukin (IL)-10, and by cell-to-cell mediated contact, and in this way can prevent autoimmune reactions.<sup>2</sup> Cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) is a surface receptor that is present on activated T effector cells. CD28, which normally binds with B7 on an antigen-presenting cell to provide the costimulatory signal necessary for T cell activation, can bind this cell surface receptor and induce T cell anergy.<sup>3</sup> On Tregs, CTLA-4 can be constitutively active, which contributes to their ability to suppress the immune system.<sup>4</sup> CTLA-4 and programmed death 1 (PD-1) are often referred to as immune checkpoints, as these 2 help form a system of checks and balances that keep immune proliferation and activation regulated. The ligand for PD-1, programmed death ligand 1 (PD-L1), engages with PD-1 to provide another signal to suppress activated CD4+ and CD8+ cells.<sup>5</sup>

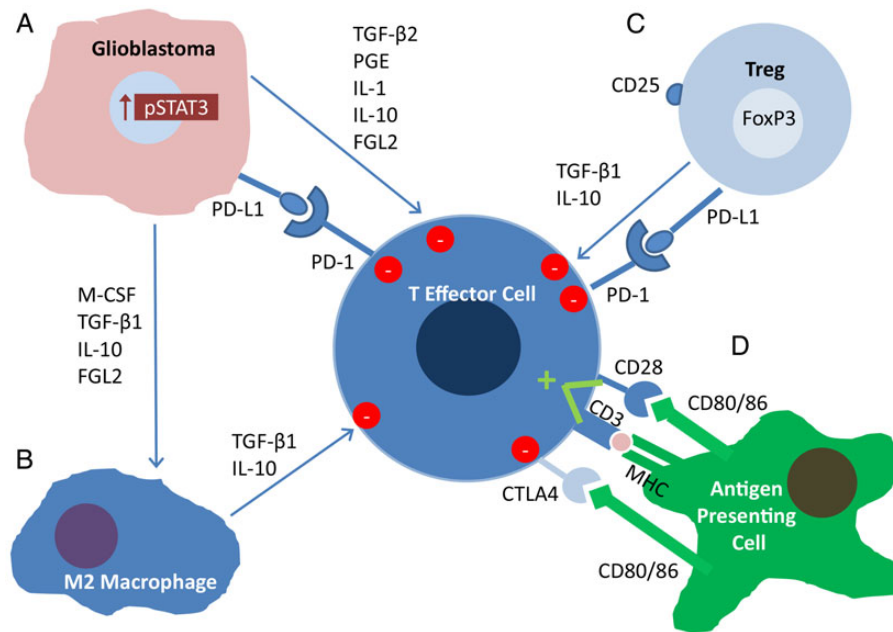
Studies of tumor microenvironments have revealed that engagement of this system is capable of inducing T cell apoptosis.<sup>6</sup>

### Secreted Immunosuppressive Factors

By examining tumor cyst fluid, it was discovered that lymphocyte activity could be suppressed using factors secreted by tumors.<sup>7</sup> These secreted factors were later identified as IL-1 and TGF- $\beta$ .<sup>8–11</sup> This discovery led to the development of trabectedin, an antisense oligonucleotide that inhibited TGF- $\beta$ 2; but despite initial promise, treatment by convection-enhanced delivery ultimately did not lead to a viable treatment strategy.<sup>12</sup> Inhibitors of TGF- $\beta$  receptor kinase activity are currently in clinical testing. Although both clinical tolerability and therapeutic activity have been limited so far,<sup>13</sup> preclinical studies support their use.<sup>14</sup> Subsequently, many other secreted factors in the glioblastoma microenvironment have been discovered, all of which have some varying level of effect on the immune response to glioblastoma. For example, colony stimulating factor 1 (CSF-1), produced by gliomas,<sup>15</sup> has been shown to polarize the macrophage to a glioma-supportive M2 phenotype, which enhances glioma progression.<sup>16</sup> Furthermore, vascular endothelial growth factor not only increases glioma tumorigenicity and growth<sup>17–20</sup> but can also inhibit the maturation and function of dendritic cells.<sup>21</sup> Other cytokines include IL-10, prostaglandin E, nitric oxide, regeneration and tolerance factor,

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**Fig. 1.** Immunosuppression in the glioblastoma microenvironment. (A) Partially driven by increased STAT3 expression, glioblastoma cells secrete immunosuppressive factors such as TGF $\beta$ -2, PGE, IL-1, IL-10 and FGL2, all of which suppress the activity of effector cells. PD-L1 expressed on its surface also engages PD-1 to suppress effector activity. M-CSF, TGF $\beta$ -1 and IL-10 skew macrophages to the immunosuppressive M2 phenotype. (B) M2 Macrophages secrete TGF $\beta$ -1 and IL-10, suppressing effector cells further. (C) Regulatory T cells secrete TGF $\beta$ -1 and IL-10 as well, further suppressing immune reactivity, while also expressing PD-L1. (D) M-CSF, TGF $\beta$ -1 and IL-10, secreted by the glioblastoma, skew tumor associated macrophages to the immunosuppressive M2 phenotype. (E) Antigen is presented to T cells by antigen presenting cells within an MHC molecule, but a costimulatory signal from CD80/86 to CD28 is required for activation. CD80/86 can also suppress activity by engaging the CTLA4 receptor on the activated T cell.

and arginase 1.<sup>22–25</sup> Regeneration and tolerance factor was found to inhibit the ability of natural killer (NK) cells to lyse target cells.<sup>26</sup> Serum arginase I levels have been found to be associated with neutrophil degranulation and immunosuppression in glioblastoma patients.<sup>27</sup> These secreted factors may be upregulated after gliomas undergo conventional therapy.<sup>28</sup> Of note, some glioma-secreted cytokines may have both immune stimulatory and inhibitory roles depending on the context. Granulocyte macrophage CSF, which functions as a cytokine and growth factor for granulocytes and monocytes, can induce myeloid-derived suppressor cells (MDSCs).<sup>29</sup>

## Glioma Cell Surface Immunosuppressive Factors

Glioma cells can express CD95 (Fas/apoptosis antigen 1) ligand on their surface, which can induce apoptosis and thus reduce the number of infiltrating T cells in the tumor microenvironment.<sup>30,31</sup> Similarly, CD70, by direct cell-to-cell mediated contact, may have an apoptotic effect on immune cells in vitro.<sup>32,33</sup> Yet, the biological effects of CD70 are context dependent, and immune stimulatory effects may dominate in vivo, at least in murine glioma models.<sup>34,35</sup> Lectin-like transcript 1 has been shown to be a glioblastoma-expressed inhibitory ligand for NK cells.<sup>36</sup> The most prototypical example is, of course, PD-L1, the ligand for PD-1, which is upregulated in the

glioblastoma microenvironment<sup>37</sup> and may be associated with the mesenchymal glioblastoma subtype.<sup>38</sup> PD-L1 is expressed on immune cells and gliomas<sup>39–42</sup> and has been shown to inhibit T cell activation and induce T cell apoptosis.<sup>6,43</sup> This upregulation in glioblastoma may be due to loss of the tumor suppressor gene phosphatase and tensin homolog.<sup>39</sup>

Enthusiasm for immune checkpoint therapeutics is based on the observed improvement in survival time of melanoma patients in phase III clinical trials,<sup>44,45</sup> which ultimately led to FDA approval of ipilimumab. Preclinical efficacy of anti-CTLA approaches in murine models of glioma<sup>46</sup> has provided support for their implementation in glioblastoma patients. Several anti-PD-1 antibodies (nivolumab, pembrolizumab) have also been clinically tested in melanoma patients,<sup>47–49</sup> with FDA approval of nivolumab and pembrolizumab in 2014.

The use of immune checkpoint inhibitors in glioblastoma patients has preclinical justification. For example, CTLA-4 blockade with coadministration of IL-12 led to tumor clearance in a murine model system, with an associated increased immune effector response and reduction in Tregs.<sup>50</sup> Furthermore, anti-PD-1 blockade has been shown to improve survival in murine glioma model systems in combination with radiotherapy.<sup>51</sup> Additionally, in a murine model of intracerebral glioma, combinatorial therapy of indoleamine 2,3-dioxygenase (IDO), CTLA-4, and PD-L1 was curative in a marked number of mice.<sup>52</sup> An alternative/supplementary approach for inhibiting Tregs is the use of the anti-IL2R $\alpha$  antibody, daclizumab, which has been

shown to decrease Treg numbers and increase the ratio of effector T cells to Tregs in patients undergoing standard-of-care treatment with temozolomide.<sup>53</sup> Cumulatively, these data provide a sufficient rationale for the use of immune checkpoint inhibitors, and a variety of clinical trials are currently under way using these strategies in glioblastoma patients.

## Immunosuppressive Cells in the Glioma Microenvironment

Glioblastoma patients can be profoundly immunosuppressed. One key feature is a general paucity of CD4-positive T cells in peripheral blood with an increased proportion of Tregs.<sup>54</sup> Chemokine C-C ligand 2, produced by glioblastoma cells, and other soluble factors<sup>55</sup> can trigger the trafficking of Tregs to the tumor microenvironment.<sup>56</sup> However, not all glioblastomas show a significant infiltration of Tregs,<sup>57</sup> suggesting a heterogeneity of immune suppressive mechanisms. The functional activity of the Tregs in the tumor microenvironment may be further enhanced by the activity of IDO, an intracellular enzyme overexpressed in many cancers, including gliomas, which suppresses T cell activation and proliferation by creating a tryptophan shortage.<sup>58</sup>

Microglia and macrophages, the latter derived from monocytes, can constitute up to 12% of the tumor mass within gliomas.<sup>59</sup> Macrophages are believed to be activated either by the classical pathway, involving bacterial products and interferon- $\gamma$  (ie, M1), or by alternative activation with T-helper 2-type cytokines such as IL-4, IL-10, IL-13, and/or macrophage CSF.<sup>60</sup> The M2 macrophages generally express CD163 and CD204 and display an immune suppressive phenotype. Glioblastomas have the highest frequency of infiltration by tumor-associated macrophages, which tend to be of the M2 lineage.<sup>61</sup> Factors produced by glioblastoma cancer stem cells may be responsible for the skew to the M2 phenotype in the glioblastoma microenvironment.<sup>62</sup> In a study of an immune modulating microRNA, miR-142-3p, it was discovered that M2 macrophages are dependent on the stimulation of the TGF- $\beta$  receptor pathway, unlike M1 macrophages.<sup>63</sup> Moreover, tumor-associated macrophages have been shown to enhance the invasiveness of glioma stem cells (GSCs) via the TGF- $\beta$ 1 signaling pathway.<sup>64</sup> MDSCs also contribute to glioblastoma-mediated immune suppression.<sup>65,66</sup> Normal monocytes exposed to glioma cells acquire properties akin to those of MDSCs.<sup>67</sup> Both galectin-1 and arginase have been tied to MDSC suppressor function.<sup>68</sup> Whereas both CD14-positive and CD15-positive MDSC subtypes have been found in the blood of glioma patients, it is the CD15 subtype that was found to be upregulated in the glioblastoma microenvironment.<sup>69</sup> Based on data from other types of malignancies,<sup>70</sup> it is likely that NK cells also play a key role in glioblastoma-mediated immune suppression, and this is an area of active investigation. An emerging area of therapeutic development has been directed toward targeting innate immunity.<sup>16</sup>

## Hubs of Tumor-Mediated Immunosuppression

Given the heterogeneity and redundancy of immune suppressive mechanisms in glioblastoma, identifying key hubs that

mediate many of these functions is theoretically appealing. Signal transducer and activator of transcription 3 (STAT3) is a transcription factor that has been found to be ubiquitously expressed in glioblastoma cells.<sup>71</sup> IL-10 activation of STAT3 in macrophages inhibits their proliferation.<sup>72</sup> Blocking STAT3 activation in the glioma microenvironment leads to an upregulation of immune-stimulatory cytokines IL-2, IL-4, IL-12, and IL-15 and of costimulatory molecules CD80 and CD86.<sup>72</sup> GSCs are known to be resistant to conventional treatment methods and are likely to be capable of restoring all of the cells within a glioblastoma after other portions of the tumor are effectively treated.<sup>73</sup> GSCs also cause immunosuppression in the glioma microenvironment by activating the STAT3 pathway and by increasing the number of Tregs.<sup>74</sup> Hypoxia further induces STAT3 and its transcriptionally regulated downstream pathways, those of hypoxia inducible factor 1  $\alpha$  and vascular endothelial growth factor.<sup>75</sup> A variety of approaches targeting STAT3 are currently in preclinical development.<sup>75–86</sup>

A second hub of glioblastoma-mediated immune suppression has recently been described. Fibrinogen-like protein 2 (FGL2) is a secreted factor that has been implicated by many different investigators as immunosuppressive. A recent study has shown that FGL2 is overexpressed in glioblastoma relative to low-grade glioma samples.<sup>87</sup> An examination of data from The Cancer Genome Atlas showed that high levels of FGL2 mRNA portended worse survival for patients than did low expression. Furthermore, higher protein levels of FGL2 were found in high-grade gliomas than in low-grade tumors. FGL2 augmented glioma immunosuppression by increasing the expression levels of PD-1, expanding the frequency of tumor-supportive M2 macrophages, and enhancing the number of MDSCs and Tregs, especially within the glioma microenvironment. Anti-FGL2 antibody treatment not only increased survival time in a murine model of intracerebral gliomas but also reduced the number of Tregs, M2 immunosuppressive macrophages, and MDSCs, and decreased the expression of PD-1. Given these data, FGL2 may be an important immunosuppressive factor that merits further targeting, specifically in glioblastoma.

## Placing Immune Suppressive Targeting into Therapeutic Context

Although there is currently great enthusiasm for the use of modulators to inhibit immune suppression, one needs to bear in mind that multiple steps are necessary for the development of an optimal antitumor immune response. First, there must be an *immunological target* (ie, antigen) present for the immune system to be directed toward. Examples of glioblastoma-specific targets and approaches include: epidermal growth factor receptor variant III, isocitrate dehydrogenase 1 mutation, and the IMA-950 glioblastoma profile panel. In the setting of minimal antigen or target expression, alternative immunotherapeutic approaches such as NK chimeric antigen receptor immunotherapy could be utilized. Second, the immune system must be *activated*. There are a variety of ways to do this, including triggering/engaging a costimulatory molecule such as 4-1BB or OX40; providing proinflammatory cytokines such as granulocyte macrophage CSF, interferon- $\gamma$ , IL-12, and IL-15;

engaging innate immunity with toll-like receptor agonists such as cytosine-phosphate-guanine or poly-inosinic:cytidylic acid; providing activated antigen-presenting cells by adoptive transfer of dendritic cells; or using stimulator of interferon gene ("Sting") agonists. Thereafter, there needs to be sufficient *traf-ficking* and infiltration into the tumor site. This may depend on tumor expression of chemokines such as CX3CR1. Finally, there must be maintenance of the immune effector function that is blocked and inhibited by tumor-mediated immune suppression. Strategies that can be employed for this function include, but are not limited to, inhibition of immunosuppression cytokines such as TGF- $\beta$  and IL-10; targeting of key hubs of multimodality immune suppression such as STAT3 and FGL2; and, the most successful thus far, use of immune checkpoint inhibitors.

As we move into the next generation of immunotherapeutic strategies, it is evident that combinatorial approaches that address each step in this continuum will be necessary to maximally impact outcomes. One needs to bear in mind that there is a great deal of plasticity within glioblastoma, and multiple operational mechanisms of immune suppression exist. When one mechanism of immune suppression is inhibited, other mechanisms can assume a dominant role. Thus, there has been an increasing interest in directing therapeutics to targets that control multiple mechanisms of tumor-mediated immune suppression. Until optimal combinatorial approaches are implemented, it is likely that glioblastoma patients will be stratified and selected for various immune therapeutic strategies based on immune biomarkers, similar to the strategies based on genomic profiling for targeted therapeutics.

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